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CELL VOLUME REGULATION BY TROUT ERYTHROCYTES: CHARACTERISTICS OF THE TRANSPORT SYSTEMS ACTIVATED BY HYPOTONIC SWELLING

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SUMMARY

1. An osmolality reduction of the suspending medium leads to osmotic swelling of trout erythrocytes, which is followed by a volume readjustment towards the original level. The regulatory volume decrease (RVD) was not complete after 1 h.

2. During RVD the cells lost K^+ and Cl^- but gained Na^+. This entry of Na^+, which is about half the K^+ loss, explains the incomplete volume recovery (it was complete when Na^+ was replaced by impermeant *N*-methyl-D-glucamine). The cells also lose large quantities of taurine, which accounts for about 53% of the volume recovery. In addition RVD is accompanied by the activation of a pathway allowing some large organic cations which are normally impermeant, such as choline or tetramethylammonium, to rapidly penetrate the cells.

3. The swelling-activated K^+ loss is not significantly affected by replacement of Cl^- by NO_3^-, indicating that K^+ moves through a Cl^--independent K^+ pathway. Furosemide, DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid) and niflumic acid inhibit the K^+ loss. From experiments performed in high-K^+-containing media, it appears that these compounds block the K^+ flux, not by inhibiting Cl^- movements but by interfering with the K^+ pathway.

4. All the volume-activated pathways (K^+, Na^+, taurine, choline) are fully inhibited by furosemide and by inhibitors of the anion exchanger such as DIDS and niflumic acid. The concentration required for 50% inhibition (IC_{50}) of both inorganic cations and taurine appears to be similar. It is proposed that DIDS interacts with a unique target which controls all the volume-sensitive transport systems.

INTRODUCTION

Perturbation of cell volume elicits alterations in membrane transport processes which act to restore the initial volume. The regulatory volume decrease (RVD) observed in hypotonically swollen cells is accomplished through the net extrusion of intracellular solutes, accompanied by osmotic water loss. In red blood cells of

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vertebrates, the types of osmolyte used during hypotonic volume regulation are species dependent. Mammalian, avian and amphibian erythrocytes rely predominantly on inorganic ions while both organic and inorganic solutes participate in teleost and elasmobranch erythrocytes (Hoffman & Simonsen, 1989; Hall, Bianchini & Ellory, 1990). The inorganic ion transport involved in this process also exhibits species specificity, but in fish (Lauf, 1982; Bourne & Cossins, 1984), duck (Haas & McManus, 1985) and human erythrocytes (Hall & Ellory, 1986; Brugnara & Tosteson, 1987; Canessa, Fabry, Blumenfeld & Nagel, 1987) it is thought to be mediated through a Cl^--dependent K^+ transport.

The present study was undertaken to characterize precisely in trout erythrocytes the different transport systems activated by the hypotonic swelling of cells and which are involved in the subsequent volume readjustment. This analysis was justified by two observations. (1) When trout red cells swell in an isotonic medium following β-adrenergic stimulation, a volume decrease response has been observed which corresponds to a Cl^--dependent K^+ loss without any apparent loss of organic solutes (Borgese, Garcia-Romeu & Motais, 1987). Thus it was of interest to check whether a loss of organic solutes participates in the regulatory volume decrease (RVD) after hypotonic swelling. (2) Preliminary experiments unexpectedly indicated that the K^+ loss occurring during hypotonic volume regulation was not Cl^- dependent.

The transport systems activated by hypotonic swelling will be compared, in a subsequent paper, with those systems involved in the β-adrenergic-induced isotonic swelling, in order to determine the factors which allow the cells to adopt one regulatory system rather than the other.

METHODS

Preparation of cells

Rainbow trout (*Salmo gairdneri*) were obtained from a commercial hatchery and kept for 1 week in the laboratory in tanks provided with running tap water (water temperature 15 °C). Fish were anaesthetized with MS 222 (Sandoz; 70 mg (l water)$^{-1}$ and blood was removed from the caudal vein by a heparinized syringe. The blood of several animals was pooled. The cells were washed three times in isotonic saline solution and the buffy coat removed by suction. They were then suspended at a haematocrit of 15% and incubated overnight at 4 °C in the saline solution to ensure that they had reached a steady state with respect to ion and water content before experimental treatment.

Experimental solutions

The isotonic saline solution contained (mM): NaCl, 145; $CaCl_2$, 5; $MgSO_4$, 1; KCl, 4; HEPPS, 5; glucose, 5; pH 7·90 (320 mosM). In some experiments internal and external Cl^- was replaced with NO_3^- by washing the cells several times in a saline containing $NaNO_3$, KNO_3 and $Ca(NO_3)_2$ instead of the chloride salts. In some other experiments the cells were suspended in medium in which Na^+ was replaced with choline, tetramethylammonium, *N*-methyl-D-glucamine or K^+. The osmolality of the media was reduced (215 mosM) by adding hypotonic saline (i.e. isotonic saline without NaCl) to the suspension at time zero; the osmolality of the media had been systematically measured with a vapour pressure osmometer (Wescor, Logan, UT, USA; model 5500). All the experiments were made in solutions thoroughly flushed with a N_2 and under a N_2 atmosphere since it has been demonstrated that oxygenation of red cells *per se* activates a Cl^--dependent K^+ pathway which remains activated for at least 1 h (Borgese, Motais & Garcia-Romeu, 1991). Ouabain and inhibitors were added immediately before hypotonic treatment.

Cell ion, amino acid and water contents

After the incubation period the red cells were washed four times in the experimental solution (15 °C). The experiments were performed at 15 °C. At intervals samples of whole suspension were poured into nylon tubes which were centrifuged at 30000 *g* for 10 min in a Sorvall RC 2B refrigerated centrifuge. These specially prepared tubes contain up to 0·17 ml.

For each time sample at least three nylon tubes were filled with cell suspension. After centrifugation the packed cell mass was separated from the supernatant by slicing the tube with a razor blade below the top of the red cell column. Cells were then prepared for analysis of cell water and ion contents.

Cell water

The packed cell mass was expressed with a close-fitting plastic rod onto weighed aluminium foil. After weighing the packed cells were dried to constant weight for 10 h at 90 °C and reweighed. Cell water content was determined in triplicate and was expressed as grams of water per gram dry cell solids.

Ion content

The dry cells were suspended in 5 ml distilled water and mixed carefully for 12 h. 200 μl of 70 % (v/v) perchloric acid was then added to the suspension. After centrifugation at 20000 *g* for 15 min the clear supernatant was saved for analysis of cations, Cl^- and amino acids. Measurements of ions were made as previously described (Baroin, Garcia-Romeu, Lamarre & Motais, 1984). A trapping correction of 3·7 % was routinely applied to the final calculation. Ion contents were expressed in micromoles per gram dry cell solids.

Amino acid content

Fifty microlitre aliquots of the 5 ml extract of the dried pellet were used to measure the concentration of the total primary amino groups by the fluorescamine assay according to Castell, Cervera & Mares (1979).

The analysis of the free amino acid contained in the erythrocytes was determined by ion exchange chromatography on sulphosalicyclic acid protein-free extracts of the dried pellets (LKB 4150 alpha Amino acid analyzer).

Choline influx

Erythrocytes were suspended in an isotonic medium in which NaCl was replaced by choline chloride. Radiolabelled choline was added to the medium (0·2 μCi ml^{-1}). Choline content was determined in triplicate at intervals during the continued incubation under isotonic or hypotonic conditions. Samples were centrifuged in nylon tubes and treated as above for cell water determination. The dry cells were suspended in 1·5 ml distilled water for 12 h. The radioactivity was measured in 1 ml extracts by liquid scintillation counting. The choline uptake (μmol (g dry cell solids)$^{-1}$ was calculated from the specific [^{14}C]choline activity in the medium and the cellular [^{14}C]choline content. A trapping correction of 3·7 % was applied.

K^+ (^{86}Rb) influx

K^+ influx was measured over a 20 min incubation period after adding 1 μCi ^{86}Rb per millilitre of suspension. The reaction was stopped by centrifugation in the nylon tube. The treatment for extraction and counting was the same as for choline. Since the ^{86}Rb uptake during the 20 min of incubation is linear, the influx was expressed as μmol (g dry cell solids)$^{-1}$ min^{-1}.

Materials

Ouabain and DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid) were obtained from Sigma (St Louis, USA). HEPPS (*N*-(hydroxy-2-ethyl)-piperazine-*N*′-propanesulphonic acid) was from Merck–Schuchardt (Darmstadt, Germany), *N*-methyl-D-glucamine-3-tetramethylammonium was from Aldrich (Germany), niflumic acid was from UPSA (France) and [^{14}C]choline was from Amersham. All other chemicals used were of reagent grade.

RESULTS

Ion, amino acid and water content of volume-static control cells

Table 1 presents the ion, amino acid and water content of control trout red cells (i.e. thoroughly washed after bleeding and then incubated overnight at 4 °C in the isotonic normal trout saline at pH 7·95). This ensured that cells were fully equilibrated and were not in a catecholamine-stimulated condition. The amino acid analysis established that the amino acid pool of these cells consisted mainly of taurine (95% corresponding to about 50 mmol (l cell water)$^{-1}$). Of the other amino acids detected, only GABA was found in measurable concentrations (about 2 mmol (l cell water)$^{-1}$).

The high intracellular concentration of taurine found in trout erythrocytes is comparable with those found in red blood cells of the European flounder (Fugelli & Zachariassen, 1976), eel (Fincham, Wolowyk & Young, 1987) and skate (Boyd, Cha, Forster & Goldstein, 1977) and in Ehrlich ascites tumour cells (Hoffmann & Hendil, 1976).

The effect of hypotonic treatment on net ion and amino acid movements

Figure 1 is a mean presentation of seventeen experiments, showing the water and solute content of experimental (○) and control cells (●) as a function of time. At time zero the isotonic saline (320 mosM) was made hypotonic (215 mosM) by addition of buffered water (see Methods). After rapid osmotic swelling to 136% of control cell volume the cells began to regulate their volume back towards initial values. The rate of water loss was initially rapid and decreased progressively. Under the experimental conditions employed, the regulatory volume decrease (RVD) was not complete, cell volume becoming stable at 116% of control cell volume in 1 h. A very similar pattern was observed in the absence of ouabain in the medium (not shown) indicating that RVD is not affected by inhibition of the Na^+–K^+ pump with ouabain.

The RVD is dependent on a reduction in the total number of cellular solutes. Figure 1 presents the ion and amino acid contents of ouabain-poisoned cells undergoing RVD. These data show that subsequent to osmotic swelling the cells showed a net loss of K^+, Cl^- and amino acids, whereas cellular Na^+ content increased. The cellular amino acid content was reduced by 50% in 1 h. There was a gradual decline in cellular amino acid and K^+ content with a similar half-time (17–18 min). Table 2 presents the mean values of solutes and water changes measured after 60 min in the seventeen experiments presented in Fig. 1. Several aspects warrant emphasis.

(1) Red blood cells lost $39{\cdot}86 \pm 1{\cdot}99$ μmol K^+ (g dry cell solids)$^{-1}$ and gained $19{\cdot}20 \pm 1{\cdot}10$ μmol Na^+ (g dry cell solids)$^{-1}$ whereas, during the same period of time, the control ouabain-poisoned cells lost only $3{\cdot}59 \pm 1{\cdot}03$ μmol K^+ (g dry cell solids)$^{-1}$ and gained $5{\cdot}31 \pm 0{\cdot}43$ μmol Na^+ (g dry cell solids)$^{-1}$. Thus cell swelling activated pathways for K^+ and Na^+ transport which increased dissipative fluxes tenfold. Similarly, the loss of amino acids (principally taurine) in control cells was undetectable and increased dramatically after exposure to the hypotonic medium ($43{\cdot}37 \pm 2{\cdot}12$ μmol (g dry cell solids)$^{-1}$. Quantitatively the losses of K^+ and taurine are closely related over the full time course of RVD (Fig. 1). The taurine lost from red cells can be quantitatively recovered from the extracellular medium (data not shown). In conclusion, enlargement of cells by hypotonic stress activates three

volume-dependent pathways allowing Na^+, K^+ and amino acids to passively diffuse across the red cell membrane. In red cells having a high resting anion conductance and a very high rate of anion exchange, the transport of Cl^- across the membrane does not have to be activated by hypotonic stress.

(2) Na^+ penetrating into the cell promotes water uptake, whereas K^+ leaving the cell promotes water loss. The amount of Na^+ penetrating the cell during RVD is

TABLE 1. Control water, ion and amino acid contents (mean ± S.E.M.; $n = 17$) of cells incubated overnight in isotonic trout saline (pH = 7·95)

	μmol (g dry cell solids)$^{-1}$	mmol (l cell water)$^{-1}$
Na^+	12·3 ± 1·50	7·1 ± 0·87
K^+	270·9 ± 2·86	156·6 ± 1·65
Cl^-	114·9 ± 3·92	66·4 ± 2·27
Amino acids	91·5 ± 3·40	52·9 ± 1·97
	g (g dry cell solids)$^{-1}$	
Water	1·73 ± 0·02	

about half the amount of K^+ leaving the cell (see Fig. 1); 50% of the K^+ loss was osmotically ineffective.

(3) The amount of positive charge (K^+, Na^+) leaving the cell was electrically balanced by an equivalent amount of chloride leaving the cell (difference $+3·52 \pm 2·53$ μmol (g dry cell solids)$^{-1}$.

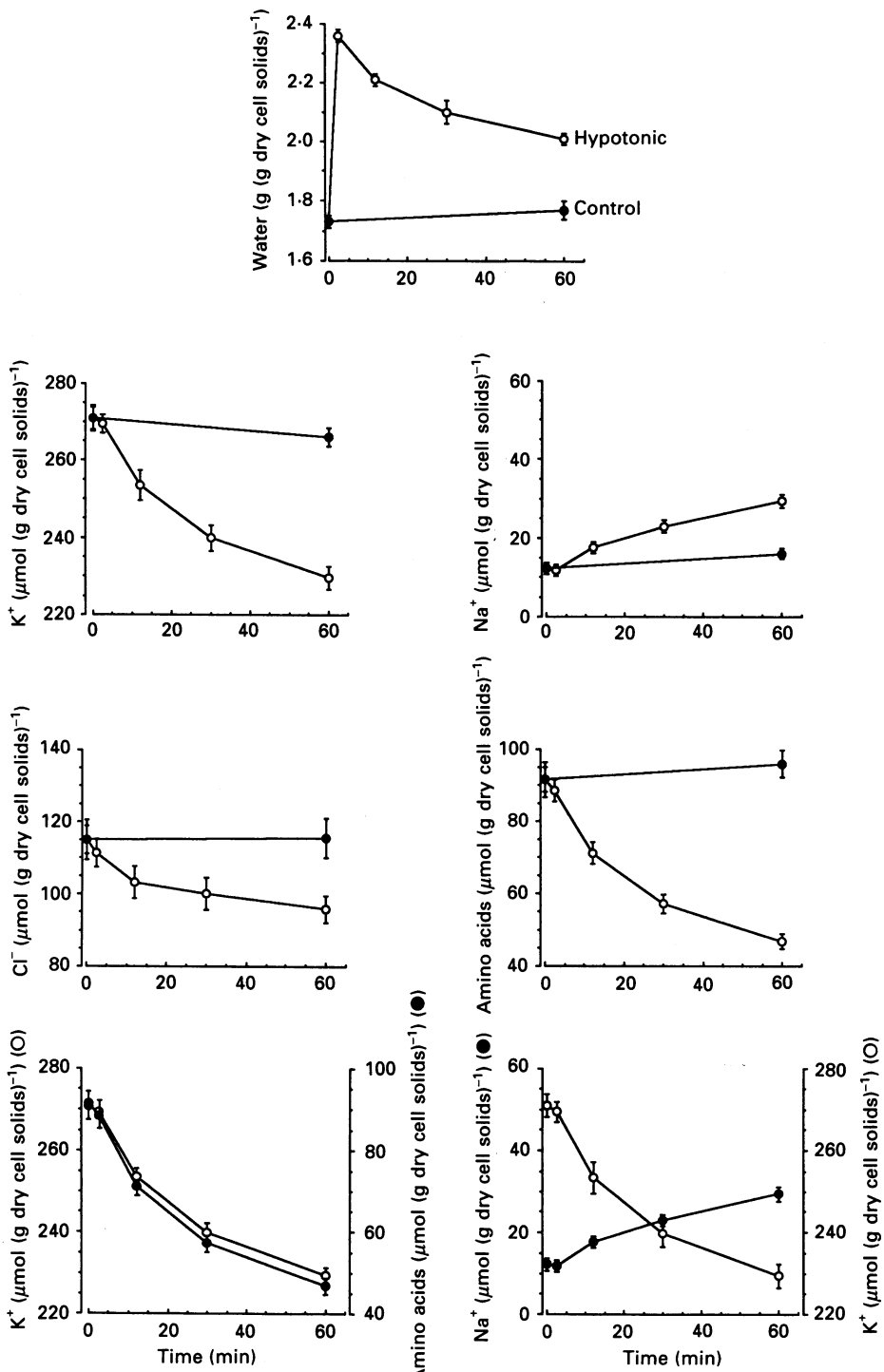
(4) The net water movement during RVD results from the net movement of osmotically active cell solutes. As shown in Table 2, the cell water calculated to be dragged by the net loss of solutes during 60 min corresponds closely with the measured loss of cell water ($0·38 \pm 0·02$ and $0·37 \pm 0·02$ g water (g dry cell solids)$^{-1}$ respectively). Due to the adverse effect of net Na^+ uptake, the net movement of ions (K^+, Cl^-, Na^+) accounted for only 46% of the water loss, the taurine efflux making up the difference.

(5) In the absence of ouabain the Na^+ penetrating into the cell was extruded and replaced by K^+ via the Na^+–K^+ pump without a significant effect on the loss of cellular water (data not shown).

Effect of Cl^- replacement on the regulatory volume decrease and K^+ loss

Because NO_3^- and Cl^- are transported at about equal rates by the anion exchanger of trout erythrocytes (Baroin *et al.* 1984; Borgese, Garcia-Romeu & Motais, 1986) the substitution of NO_3^- for Cl^- should not change the rate of the RVD if cation movements are mediated by a Cl^--independent transport system.

Figure 2*A* illustrates the changes in water content as a function of time when cells are hypotonically swollen either in a chloride medium or in a nitrate medium where both intra- and extracellular Cl^- were replaced by NO_3^-. These data, which represent the mean of five pairs of experiments, show that RVD was significantly inhibited in NO_3^- saline. Thus, 60 min after the hypotonic stress the water loss was $0·38 \pm 0·02$ and $0·27 \pm 0·03$ g water (g dry cell solids)$^{-1}$ in Cl^- and NO_3^- media, respectively (difference $0·11 \pm 0·03$; $P < 0·05$). The results presented in Fig. 2*B* clearly show,


Water (g (g dry cell solids)$^{-1}$)
2·4
2·2
2·0
1·8
1·6
Hypotonic
Control
0
20
40
60
K$^+$ (μmol (g dry cell solids)$^{-1}$)
280
260
240
220
Na$^+$ (μmol (g dry cell solids)$^{-1}$)
60
40
20
0
Cl$^-$ (μmol (g dry cell solids)$^{-1}$)
140
120
100
80
Amino acids (μmol (g dry cell solids)$^{-1}$)
100
80
60
40
K$^+$ (μmol (g dry cell solids)$^{-1}$) (O)
Amino acids (μmol (g dry cell solids)$^{-1}$) (●)
Time (min)
Na$^+$ (μmol (g dry cell solids)$^{-1}$) (●)
K$^+$ (μmol (g dry cell solids)$^{-1}$) (O)
Time (min)

however, that the mean K^+ loss was only slightly affected by the replacement of Cl^- by NO_3^-. As shown in Table 3, the reduction of K^+ loss by NO_3^- substitution in fact widely varies from one experiment to another: the replacement of Cl^- by NO_3^- did not at all modify K^+ loss in two experiments, a 20% reduction was observed in two others and the reduction reached 58% in one experiment. Thus it can be concluded that the bulk of K^+ loss observed after osmotic swelling is mediated by a Cl^--independent pathway but that a varying Cl^--dependent component can be observed. The substitution of NO_3^- for Cl^- does not significantly affect the loss of taurine ($-37{\cdot}97 \pm 3{\cdot}55$ and $-43{\cdot}34 \pm 3{\cdot}09$ μmol (g dry cell solids)$^{-1}$ h^{-1} respectively). Conversely it induces a large increase in Na^+ uptake of up to 60% ($18{\cdot}46 \pm 2{\cdot}59$ and $29{\cdot}10 \pm 1{\cdot}59$ μmol (g dry cell solids)$^{-1}$ h^{-1} in Cl^- and NO_3^- media respectively; difference $10{\cdot}66 \pm 3{\cdot}68$; $P < 0{\cdot}05$). Thus, in NO_3^- media the K^+ loss is only slightly reduced but the Na^+ uptake becomes almost as large as the K^+ loss. Since the cell cation content ($Na^+ + K^+$) remained almost constant during RVD (Fig. 2*C*), the rate of water loss was reduced (Fig. 2*A*) and largely accounted for by the loss of taurine.

In brief, on the basis of the criteria normally used (e.g. K^+–Cl^- co-transport does not carry NO_3^-), the swelling of trout red cells induced by hypotonic medium does not activate K^+–Cl^- co-transport but essentially a Cl^--independent K^+ pathway.

Inhibition of the volume-induced transport pathways involved in the regulatory volume decrease

The uptake of Na^+ was not inhibited by the inhibitors of the Na^+–H^+ antiport, amiloride (10^{-3} M) or EIPA (5-(*N*-ethyl-*N*-isopropyl)-amiloride, 10^{-4} M) (data not shown). In some cell types the activation of a Cl^--independent K^+ pathway by cell swelling can result from the stimulation of a Ca^{2+}-activated K^+ channel which is susceptible to inhibition by quinine (Hoffmann & Simonsen, 1989). However, no effect of this compound was observed on the RVD in trout erythrocytes (data not shown). Furthermore, when red cells were suspended in a hypotonic medium containing EGTA and no Ca^{2+}, the volume-regulatory loss of K^+ and amino acids was unaffected.

Hydrophobic quaternary ammonium ions such as tetrapentylammonium (1 mM), which react with some voltage-dependent K^+ channels (Hille, 1984), do not affect the volume-sensitive K^+ flux (data not shown).

The loop diuretic furosemide, which is known to inhibit K^+–Cl^- co-transport in different cell types when used at relatively high concentrations (see Hoffmann & Simonsen, 1989, for review), inhibits K^+ flux in hypotonically swollen trout red cells (both unidirectional K^+ influx (Fig. 3 and Bourne & Cossins, 1984) and net K^+ loss (data not shown)). Furosemide, however, is also known to inhibit the anion exchanger in mammalian red cells (Brazy & Gunn, 1976; Cousin & Motais, 1976). Thus we tested more-specific inhibitors of the red cell anion-exchange system, such as niflumic acid (Cousin & Motais, 1979) and DIDS; they also inhibited K^+ flux

Fig. 1. Water, Na^+, K^+, Cl^- and amino acid contents of trout red cells undergoing regulatory volume decrease after hypotonic swelling in the presence of 10^{-4} M-ouabain (○), and of control cells exposed to ouabain (●). The two bottom graphs show the comparative evolution of K^+, Na^+ and amino acid contents. Mean values of seventeen experiments ± S.E.M.

TABLE 2. Water (observed/expected) and solute (ion, amino acid) content changes* at 60 min in trout erythrocytes exposed to hypotonic medium ($n = 17$)

Na^+	K^+	$Na^+ + K^+$	Cl^-	$(Na^+ + K^+)-(Cl^-)$	Amino acids	Total osmotic particles	Water (measured)	Water (expected)†
μmol (g dry cell solids)$^{-1}$							g (g dry cell solids)$^{-1}$	
$+19{\cdot}20 \pm 1{\cdot}10$	$-39{\cdot}86 \pm 1{\cdot}99$	$-20{\cdot}65 \pm 1{\cdot}88$	$-17{\cdot}14 \pm 1{\cdot}73$	$+3{\cdot}52 \pm 2{\cdot}53$	$-43{\cdot}37 \pm 2{\cdot}12$	$-80{\cdot}69 \pm 4{\cdot}39$	$-0{\cdot}37 \pm 0{\cdot}02$	$-0{\cdot}38 \pm 0{\cdot}02$

* When cells shrink they lose KCl and amino acids (and gain NaCl) so they lose dry cells solids. The number of cells per gram of dry cell solids would be expected to increase during cell shrinkage, so dry cell solids may not be an appropriate unit for comparing cell contents before and after shrinkage. From the above data, however, it can be calculated that the given value after 1 h shrinkage is overestimated only by 0·7%.

† The hypotonic medium being 215 mosM and the loss of osmolytes being accompanied by an isosmotic loss of water, 4·65 ml water is lost for each mmole of osmolyte leaving the cell.

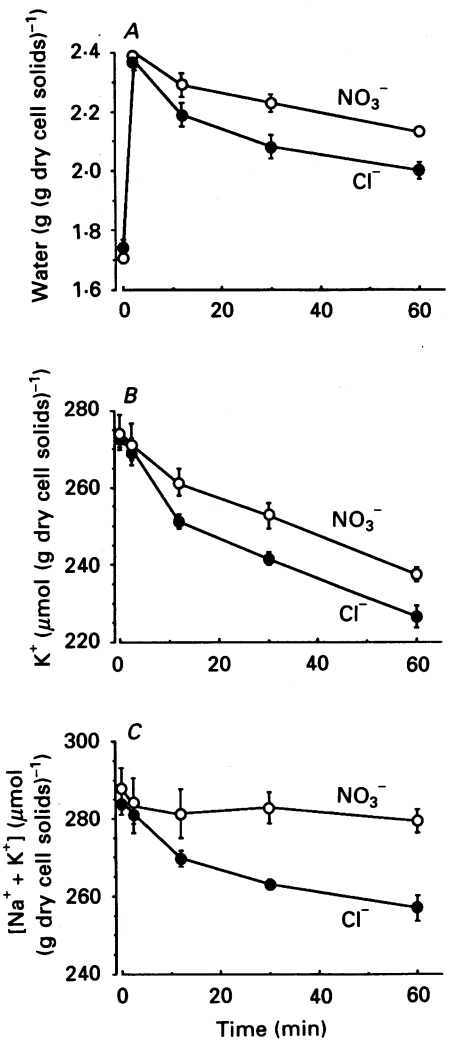


Fig. 2. Effect of NO_3^- substitution on RVD (*A*), K^+ content (*B*) and cationic content (*C*). Mean values of five paired experiments ± S.E.M.

TABLE 3. Effect of Cl^- replacement by NO_3^- on K^+ loss

	K^+ (μmol (g dry cell solids)$^{-1}$) for 60 min		
Experiment	Cl^- medium	NO_3^- medium	Differences
1	−44·06	−42·77	−1·29
2	−46·55	−36·20	−10·35
3	−39·58	−38·37	−1·21
4	−46·70	−19·40	−27·30
5	−40·47	−32·33	−8·14
Mean ± S.E.M.	−43·47 ± 1·67	−33·81 ± 4·45	−9·66 ± 5·34*

* $0{\cdot}2 > P > 0{\cdot}1$.

(unidirectional influx (Fig. 3) and net loss as shown in Fig. 4 for DIDS). As illustrated in Fig. 4 for DIDS, these compounds completely blocked RVD. This effect on volume change resulted not only from an inhibition of the loss of KCl but also from an inhibition of the loss of taurine and of the Na^+ uptake. The remaining Na^+ uptake

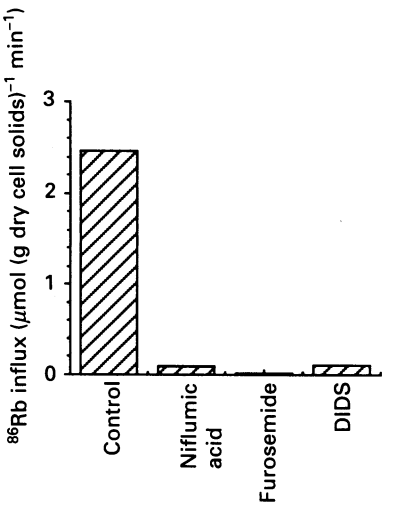


Fig. 3. Effect of furosemide (5×10^{-4} M), DIDS (10^{-4} M) and niflumic acid (5×10^{-4} M) on ^{86}Rb influx, as an estimate of the swelling-induced K^+ flux. Experiment was performed immediately after osmolarity reduction of a high-K^+-containing medium (145 mM-K^+). Similar inhibitory effects were observed in a normal Na^+-containing saline, with 4 mM-K^+. The drugs were added to the red cell suspension just before osmotic shock. Ouabain was present at 10^{-4} M.

observed in the presence of DIDS (Fig. 4) was equivalent to the entry of Na^+ measured in volume-static cells poisoned with ouabain (i.e. $5{\cdot}31 \pm 0{\cdot}43$ μmol (g dry cell solids)$^{-1}$; $n = 12$). Thus all the transport pathways expressed as a consequence of cell enlargement were inhibited by DIDS and other inhibitors of the anion exchanger.

The dose–response curves for the effect of DIDS upon K^+ and amino acid volume-sensitive fluxes are presented in Fig. 5. The curves were superimposable. The half-maximal inhibitory concentration of DIDS to inhibit both K^+ and amino acids with a 20% haematocrit was $\sim 5{\cdot}5 \times 10^{-5}$ M.

Effect of Na^+ replacement on the regulatory volume decrease

As illustrated in Fig. 6, when trout red cells were exposed to a hypotonic Na^+-free medium (choline as substitute for Na^+), the regulatory volume decrease was partially inhibited, indicating that the water loss was dependent on the type of cation present in the extracellular medium. The net loss of cellular K^+ and amino acids, however, was similar or slightly greater in choline than in sodium media (Fig. 6). Because sodium no longer penetrates into the cell, this inhibition of the water loss was surprising. Since in this experimental condition the chloride loss was fully inhibited (Fig. 6), the only way to account for both a net efflux of positive charges (K^+ ions) and a reduced loss of water was to postulate the entry of an external cation which

electrically balances the K^+ loss. Using radioactive choline it was indeed possible to demonstrate that choline, which does not enter the cell in isotonic conditions, penetrates the cell very rapidly after hypotonically induced swelling (Fig. 7*A*). In fact the net uptake of choline was equivalent to the net K^+ loss since the net

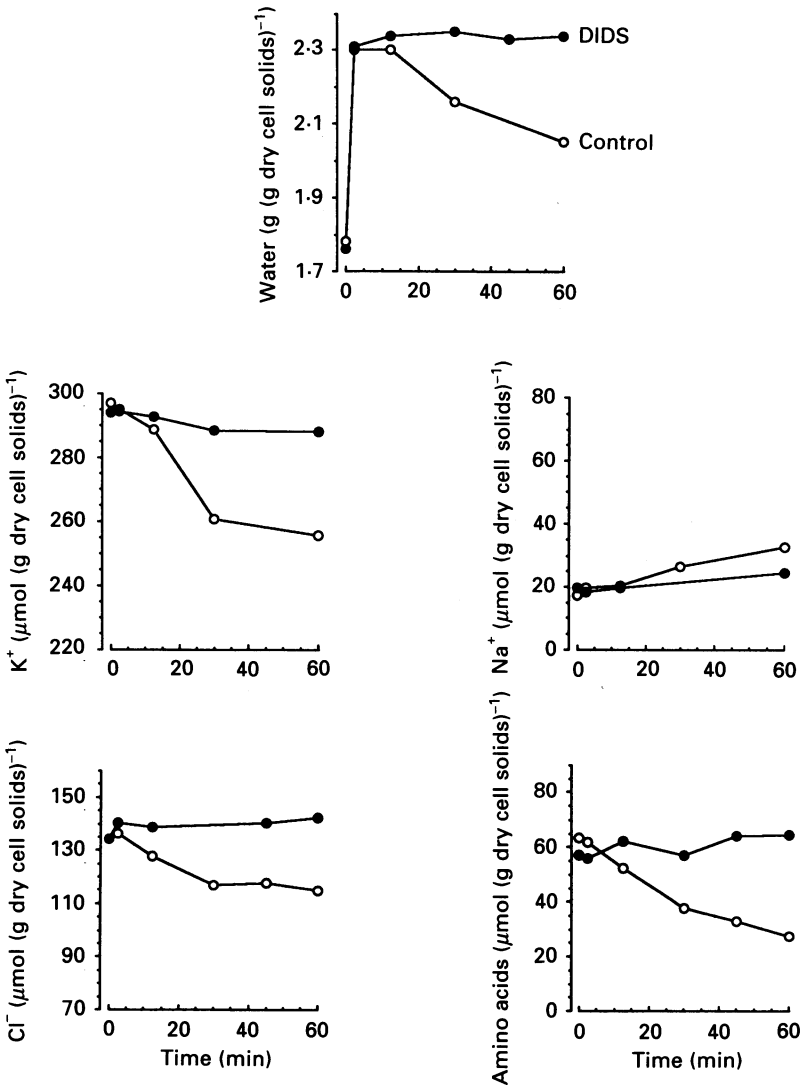


Fig. 4. Inhibitory effect of DIDS (5×10^{-4} M) on RVD, K^+, Na^+, Cl^- and amino acid movements after hypotonic swelling. DIDS was added to the suspension medium immediately before the osmotic shock. ●, DIDS; ○, control.

movement of chloride was zero. In the absence of any net movement of osmotically active charged particles, the water loss must have resulted, therefore, from the loss of amino acids. It is noteworthy that in choline medium the rate of K^+ and amino acid efflux did not decrease with time as in sodium medium, such that after 90 min

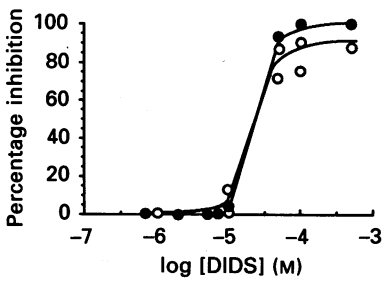


Fig. 5. Dose–response curve for DIDS inhibition of K^+ and amino acid movements during RVD. The values represent the percentage inhibition of K^+ (○) and amino acid (●) net movements compared to the control value (i.e. without inhibitor).

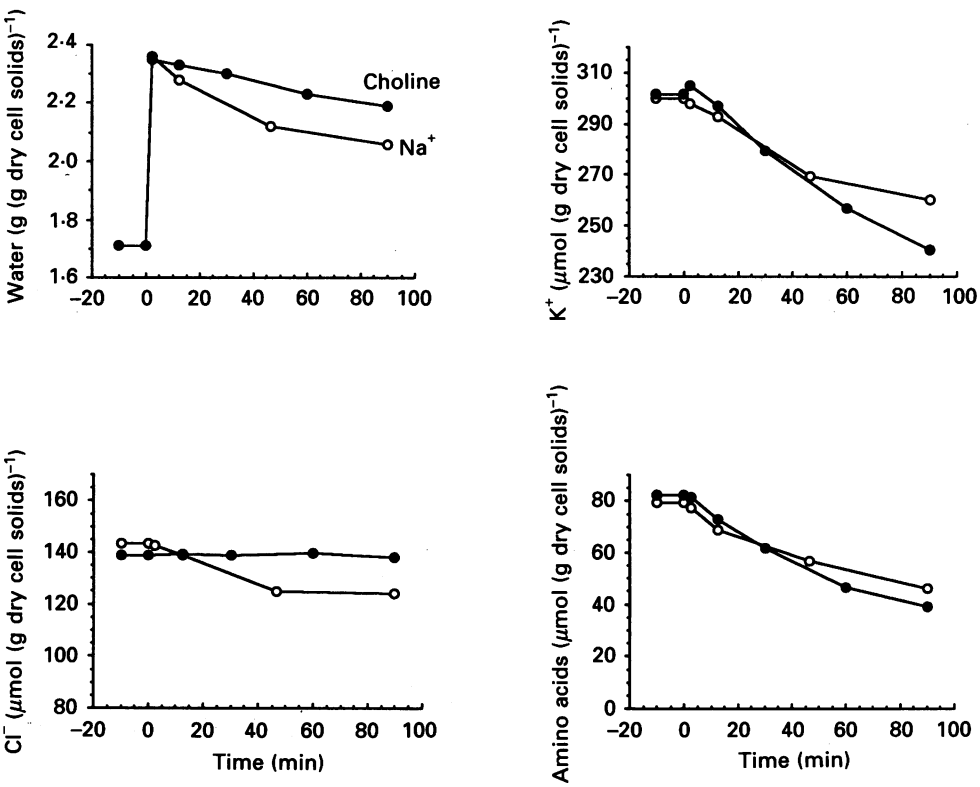


Fig. 6. Water, K^+, Cl^- and amino acid contents of cells undergoing RVD after osmotic swelling in normal Na^+-containing saline (○) and in Na^+-free choline medium (●). All the media contain ouabain (10^{-4} M).

RVD the cells had lost significantly more K^+ and amino acids in choline than in the Na^+ medium (Fig. 6). This might be due to a more sustained activation of K^+ and amino acid pathways because of the slower volume recovery.

As illustrated in Fig. 7*A* the swelling-activated choline pathway was inhibited by DIDS (5×10^{-4} M). Since K^+ and amino acid transports are also inhibited by DIDS, the RVD is completely blocked in the presence of DIDS (Fig. 7*B*). Hemicholinium, a

compound which inhibits choline transport in human red cells (Martin, 1977), had no effect on the swelling-induced choline pathway (data not shown).

When red cells were exposed to hypotonic medium in which Na^+ was replaced not by choline but by another presumed impermeant cation such as tetramethyl-

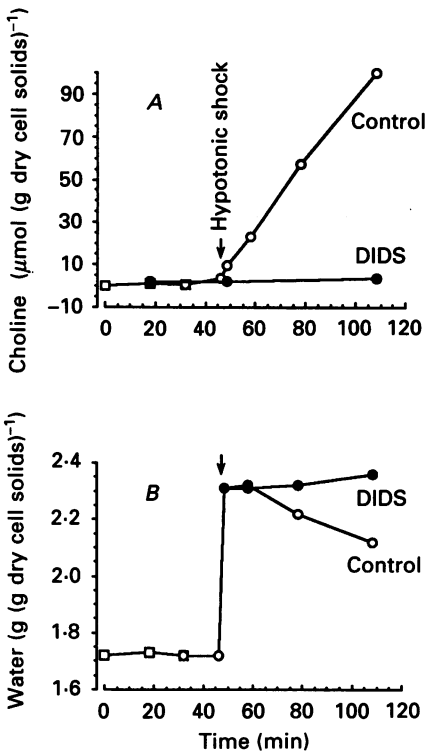


Fig. 7. *A*, uptake of choline as a function of time with (●) or without (○) DIDS (5×10^{-4} M). Choline uptake was measured with [^{14}C]choline in a Na^+-free saline (NaCl replaced by choline chloride) as described in Methods. Osmolality was reduced by dilution at 45 min. *B*, inhibitory effect of DIDS on RVD in choline saline. ○, control cells without DIDS; ●, in the presence of 5×10^{-4} M-DIDS.

ammonium (TMA), RVD was also partially inhibited. This is despite the absence of net Na^+ uptake and the fact that amino acids and K^+ are lost, the leak of K^+ being even greater than in the Na^+-containing medium (Fig. 8). Because the chloride content in the red cell remained constant or increased slightly during RVD, it is likely that a very fast uptake of TMA occurs which electrically balances the K^+ loss. DIDS completely blocks the regulatory loss of water (not shown) suggesting that it inhibited TMA uptake as well as K^+ and amino acid loss.

The inhibitory effect of DIDS on choline and TMA uptake means that the dramatic increase in permeability to quaternary ammonium derivatives observed in hypotonic medium cannot be attributed to non-specific membrane damage resulting from cell expansion. This view is reinforced by the fact that another organic cation, *N*-methyl-D-glucamine (NMDG), cannot enter the cell. Indeed, as illustrated in Fig. 9, when the cells were suspended in a hypotonic medium in which Na^+ was

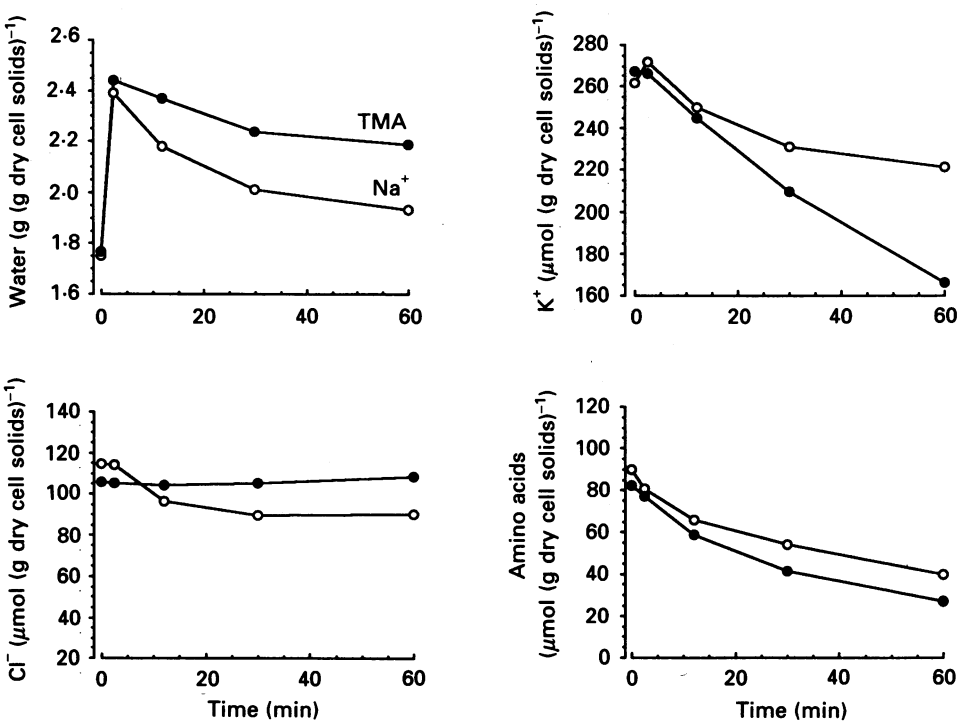


Fig. 8. Water, K^+, Cl^- and amino acid contents of cells undergoing RVD after osmotic swelling in normal Na^+-containing saline (○) and in Na^+-free saline in which NaCl was replaced by tetramethylammonium chloride (●).

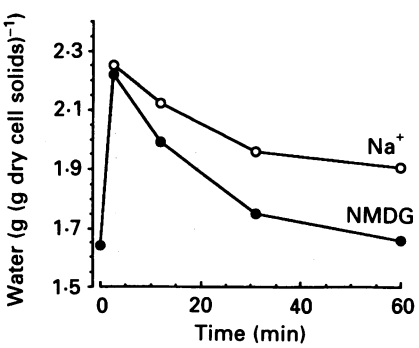


Fig. 9. Changes in cell water content as a function of time after osmotic swelling in normal Na^+-containing medium (○), and in Na^+-free saline in which Na^+ was replaced by *N*-methyl-D-glucamine (●).

replaced by NMDG a large volume-regulatory decrease occurred which was accounted for by the leak of amino acids, K^+ and Cl^- (not shown). In this experimental condition RVD is complete, i.e. in 1 h the cell volume had recovered its initial value. This is in contrast to the situation in normal Na^+-containing saline where the regulated cell volume plateaus at 116% of its initial value because of net Na^+ entry (Fig. 1). Thus the entry into the cell of NMDG is unlikely.

In conclusion, the cell swelling induced by a hypotonic medium activates a DIDS-sensitive transport pathway which accepts some normally impermeant organic cations such as choline or TMA, but rejects some other more hydrophilic compounds such as NMDG.

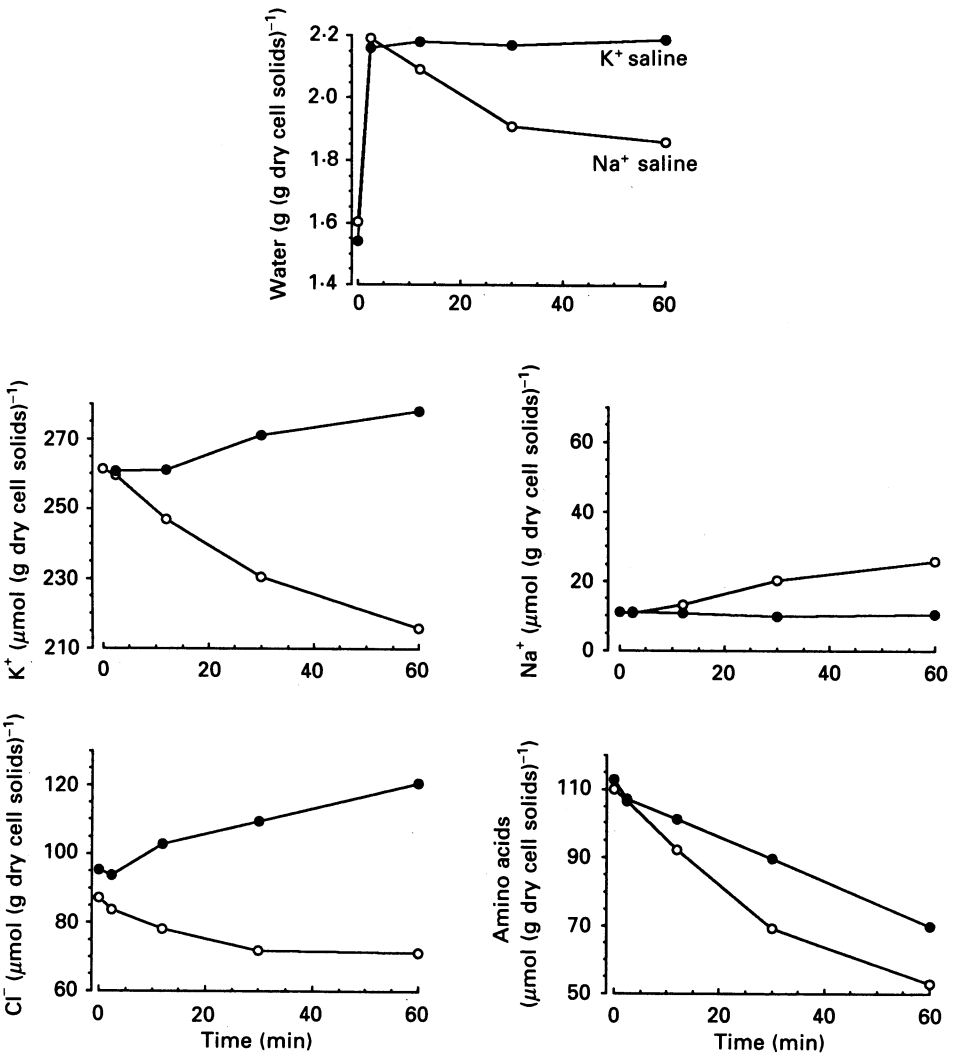


Fig. 10. Water and solute contents of cells undergoing RVD after hypotonic swelling in a normal Na^+-containing medium (○) and in a Na^+-free, high-K^+-containing medium (●).

Figure 10 shows the cellular water and solute content as a function of time when red cells were transferred to a hypotonic medium in which Na^+ was totally replaced by K^+. Whereas RVD occurred in Na^+ medium (cell volume decreased from 2·19 g water (g dry cell solids)$^{-1}$ at 2·5 min to 1·86 at 60 min), it was completely blocked in the high-K^+ medium, and even the red cell volume increased slightly (from 2·16 to

2·19 g water (g dry cell solids)$^{-1}$). This occurred despite the loss of substantial quantities of taurine from the cells. Figure 10 shows that the loss of amino acids (42·8 μmol (g dry cell solids)$^{-1}$ in 60 min) was practically osmotically balanced by a net entry of K^+ (28·21 μmol (g dry cell solids)$^{-1}$) and the accompanying anion, Cl^- (26·84 μmol (g dry cell solids)$^{-1}$). The net entry of K^+ occurred along the electrochemical gradient of the ion (at 2·5 min $[K^+]_o = 106$ mequiv l^{-1}, $[K^+]_i =$ 121 mequiv (l cell water)$^{-1}$; membrane potential $= -23$ mV calculated from the Cl^- ratio).

DISCUSSION

Respective importance of ions and amino acids in the regulatory volume decrease

Unlike mammals and birds, the red blood cells of fish possess high intracellular concentrations of amino acids. Quantitatively the most important component is taurine, an amino acid which plays a role in cell volume regulation in vertebrates (Forster & Goldstein, 1979) as well as invertebrates (Pierce, 1981). Thus for trout red cells it was not unexpected that the regulated volume reduction resulted not only from net dissipative movements of inorganic ions but also of amino acids. Concerning inorganic ions, RVD involved the loss of K^+ and Cl^- but a significant gain of Na^+. This entry of Na^+ offsets the losses of other solutes and accounts for the incomplete recovery of cell volume in a Na^+-containing medium. Indeed, cell volume recovers its initial value when external Na^+ is replaced by an impermeant cation such as NMDG (Fig. 9). Thus despite the relatively high net ionic movements, their role in the reduction of cell volume was limited: the decrease in amino acid content, essentially taurine, accounted for approximately 53% of the total reduction in cellular osmolytes occurring during the volume regulatory response, the remainder being contributed by the net loss of ions (Table 2).

A similar volume-dependent increase in taurine permeability has been observed in erythrocytes from the European flounder (Fugelli & Riersen, 1978), eel and starry flounder (Fincham *et al.* 1987), the skate (Leite & Goldstein, 1987) and in Ehrlich ascites tumour cells (Hoffmann & Lambert, 1983), but the loss of taurine in these cells accounts for a smaller fraction of the osmotic response (one-eighth to a third). Our results suggest, as previously indicated by Fincham *et al.* (1987), that this taurine efflux induced by osmotic swelling involves a Na^+- and Cl^--independent transport mechanism since it occurs in NO_3^- medium (Fig. 2) and is not accompanied by a loss of sodium in Na^+-free medium. In addition we demonstrate that it is fully inhibited by DIDS, niflumic acid, furosemide and other inhibitors of the anion exchanger. Recently, a DIDS-sensitive taurine efflux in skate red blood cells exposed to hypotonic medium has been described (Goldstein, Brill & Freund, 1989).

The net Na^+ uptake during RVD is quite unusual. In other cells undergoing RVD, it seems not to occur, except in the red blood cells of *Amphiuma* (Siebens & Kregenow, 1984) and flounder (Cala, 1977). Recently, however, Livne & Hoffmann (1990) demonstrated that RVD in Ehrlich ascites tumour cells involves not only a loss of KCl but also a cytoplasmic acidification. This acidification may activate the Na^+–H^+ exchange, the resulting gain of Na^+ leading to an incomplete RVD. This interpretation is supported by the fact that amiloride, which inhibits Na^+–H^+ exchange, enhances the RVD response. However, in trout erythrocytes we have

shown that the gain of Na^+ by cells undergoing RVD is insensitive to amiloride (10^{-3} M) and to EIPA (10^{-4} M), both of which inhibit Na^+–H^+ exchange, but is completely inhibited by DIDS. This strongly argued against the involvement of the Na^+–H^+ exchange system in the net gain of Na^+ observed during RVD in trout red blood cells.

It has been shown, in several types of cell, that hypotonic swelling activates volume-regulatory leak pathways which, by reducing the net cellular content of osmotically active particles, leads to an adaptive cell shrinking. But swelling of trout erythrocytes not only activates specific pathways for K^+, Na^+ and amino acids, which are the solutes generally involved in volume-regulatory processes, but also allows some large organic cations which are normally impermeant, i.e. choline and TMA, to cross the cell membrane. This observation rationalizes the observation by Fugelli & Thoroed (1986) of slower regulatory volume responses of flounder erythrocytes when suspended in a hypotonic medium in which NaCl was replaced by choline chloride. Moreover, these authors also noticed in the presence of choline chloride a slower delay of net taurine efflux, a phenomenon that is also noticeable for K^+ and amino acids in trout red cells. It can be accounted for by the relatively slow rate of recovery of cell volume.

In conclusion, cell swelling induced by a hypotonic stress causes the activation of several pathways allowing different solutes (K^+, Na^+, taurine, organic cations such as choline) to passively diffuse across the cell membrane. All of these transport systems can be fully inhibited by DIDS, niflumic acid and furosemide. A key question is the nature of these different pathways.

Characteristics of the volume-sensitive K^+ flux

Several K^+ transport mechanisms have been proposed to be activated during RVD: (a) an electroneutral K^+–Cl^- co-transport, (b) uncoupled K^+ and Cl^- conductive channels and (c) a K^+–H^+ exchange functionally coupled with anion exchange. According to Hall *et al.* (1990) the K^+–Cl^- co-transport system is effective for RVD principally in cells with a high resting Cl^- conductance and low membrane potential (trout hepatocytes, red cells). Conversely cells with a low Cl^- permeability rely on separate conductive channels (lymphocytes, ascite tumour cells). The distinction between these different mechanisms is based on several criteria.

K^+–Cl^- co-transport is generally considered unable to carry NO_3^- which, by contrast, is transported effectively by the anion exchanger and the anion conductive pathway. Thus, it is generally assumed that the K^+ loss is mediated by a co-transport system if K^+ movement is blocked when cell and medium chloride is replaced by NO_3^-. It should be noted, however, that this demonstration of Cl^- dependence is not unequivocal evidence of a co-transport system since the activation of different exchangers (Na^+–H^+, K^+–H^+) is also inhibited by nitrate substitution (Parker, 1983; Borgese *et al.* 1986; Adorante & Cala, 1987; Guizouarn, Scheuring, Borgese, Motais & Garcia-Romeu, 1990). The present experiments have demonstrated that the volume recovery after osmotic swelling is 30% inhibited by the substitution of chloride by nitrate in cell and medium. This partial inhibition, however, was not explained by an inhibitory effect of NO_3^- on K^+ loss, which is only slightly affected, but by a large increase in net Na^+ entry. Thus the K^+ loss induced by the hypotonic

swelling of trout red cells is clearly Cl^- independent. This result, which argues against the involvement of K^+–Cl^- co-transport, was unexpected for three reasons.

Firstly, as outlined above, the volume-stimulated K^+ flux measured in all the red blood cells studied so far is a Cl^--dependent K^+ flux considered to be mediated by K^+–Cl^- co-transport (Hall *et al.* 1990). The only exception known was *Amphiuma* red cells, in which the volume-sensitive K^+ flux was considered to be mediated by a K^+–H^+ antiport which, however, was NO_3^- sensitive (Cala, 1980, 1983). Very recently, however, Dickman & Goldstein (1990) have shown that skate red cells regulate their volume after hypotonic swelling by inducing a Cl^--independent K^+ mechanism.

Secondly, when the swelling of trout red blood cells was induced, not by exposure to hypotonic medium but by catecholamine stimulation in an isotonic medium, a volume-sensitive K^+ efflux was activated which was fully blocked when Cl^- was by NO_3^- (Baroin *et al.* 1984; Borgese *et al.* 1986). These combined observations indicate that both Cl^--dependent and Cl^--independent K^+ transport systems exist in trout red blood cells and both may play a role in volume regulation. One major difference in the conditions of activation of the two systems is that the Cl^--dependent K^+ flux was observed after stimulation of adenylate cyclase, whereas the activation of the Cl^--independent K^+ flux did not involve cyclic AMP production.

Thirdly in volume-static trout erythrocytes, a Cl^--dependent, furosemide- and DIDS-sensitive unidirectional K^+ influx has been described (Bourne & Cossins, 1984). An increase in cell volume led to a stimulation of a furosemide-sensitive K^+ influx and it was implicitly accepted that this volume-dependent component was also Cl^- dependent. It now seems likely that the Cl^--dependent K^+ flux observed in volume-static cells appears when erythrocytes, made anoxic by sedimentation during overnight equilibration, are oxygenated by resuspensions in the experimental medium since oxygenation of haemoglobin activates a Cl^--dependent K^+ transport (Borgese *et al.* 1991).

A major problem in characterizing the volume-sensitive K^+ transporter is the lack of specific inhibitors. The volume-sensitive K^+ loss in trout red cells was inhibited by furosemide, a compound known to inhibit in the same range of concentrations both K^+–Cl^- co-transport and the erythrocyte anion exchanger. In fact the volume-dependent K^+ loss in trout red cells was inhibited only by inhibitors of the erythrocyte anion exchanger: furosemide, DIDS, SITS and niflumic acid. For DIDS the concentration required for 50% inhibition, IC_{50}, is $5{\cdot}5 \times 10^{-5}$ M. Thus the present results (Cl^- independence, DIDS inhibition) leave open the possibility that K^+ transport might involve either a K^+–H^+ countertransport coupled to Cl^-–OH^- exchange or two separate conductive K^+ and Cl^- transport pathways.

It is theoretically possible to discriminate between these mechanisms either by observing changes in membrane potential following inhibition of Cl^- conductance by DIDS or by observing a transmembrane pH gradient also in the presence of DIDS. However, the results obtained in the virtual absence of net K^+ and Cl^- movements (i.e. in high-K^+-containing medium) show that such an approach is inappropriate and that the situation is more complex. In the high-K^+-containing medium, the major part of K^+ movement corresponds to an entry of K^+ ions compensated by an efflux of K^+, whatever the involved mechanism: K^+–K^+ exchanges through a

conductive K^+ channel, or K^+–H^+ *versus* H^+–K^+ exchanges mediated by a K^+–H^+ antiporter. Thus the inhibition of the anion exchanger or of the conductive Cl^- channel with DIDS is expected to leave the K^+ flux unaffected. But we found in this particular condition that DIDS and niflumic acid totally inhibit K^+ unidirectional flux (Fig. 3), strongly suggesting that DIDS interferes with the K^+ transport system itself. Similarly when cells are suspended in a choline medium, the loss of K^+ being compensated by a gain of cholinium ions, the inhibition of chloride movements by DIDS is expected to leave the K^+ and choline fluxes unaffected if conductive pathways are involved. But in this case, again, both K^+ and choline fluxes were fully inhibited by DIDS. The interference of DIDS and other anionic inhibitors with the K^+ transport system raises two problems. First it prevents a discrimination between the two postulated pathways. It is noteworthy that, to date, neither a K^+–H^+ exchanger nor a conductive K^+ pathway has been considered to be DIDS sensitive. Second, the inhibition of volume-sensitive K^+ flux by DIDS has been described previously, but only rarely and always in red blood cells: essentially in erythrocytes from toadfish (Lauf, 1982), duck (Lytle & McManus, 1987), sheep (Ellory & Dunham, 1980) and trout (Baroin *et al.* 1984; Bourne & Cossins, 1984). In all of these examples the volume-sensitive K^+ flux was Cl^- dependent (NO_3^- sensitive) and considered to be mediated by a K^+–Cl^- co-transport system, and the DIDS inhibition could be assumed to occur from an interaction of the drug with the anionic site on the transporter. It is noteworthy, however, that to our knowledge DIDS has never been shown to inhibit K^+–Cl^- co-transport systems in tissues other than erythrocytes.

In conclusion, Cl^--dependent and Cl^--independent K^+ pathways co-exist in trout erythrocytes; both are inhibited by DIDS and other inhibitors of the anion exchange and play a role in volume regulation. The Cl^--dependent K^+ flux, which may be mediated by a K^+–Cl^- co-transport system, occurs when the cells are enlarged in an isotonic medium by catecholamines and cyclic AMP. Conversely, the Cl^--independent K^+ flux is activated by the swelling of cells suspended in a hypotonic medium. The nature of this K^+ transport pathway remains obscure but DIDS appears to interfere with this K^+ pathway.

Relationships between the different volume-sensitive pathways

Hypotonic swelling of trout erythrocytes not only activates a K^+ transport pathway but also other volume-sensitive pathways allowing a passive diffusion of amino acids and Na^+. The systems which, on swelling, selectively lose K^+ and taurine, the major free osmotic particles in cells, clearly have a volume-regulatory function. The entry of Na^+, conversely, has an opposite effect. Moreover, after substitution of choline or TMA for Na^+, the enlarged cells become highly permeable to these 'unphysiological' compounds which are normally impermeant.

Surprisingly, DIDS, and other inhibitors of the anion exchanger, inhibit the movement of all of these solutes, and the IC_{50} for inhibition of both cations and taurine appears similar. It seems unlikely, however, that all of these compounds use the same pathway. Thus the most likely interpretation is to suggest that DIDS interacts with a unique target which controls all the volume-sensitive transport systems.

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